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## Anti-tumor activity of tanshinone IIA in combined with cyclophosphamide against Lewis mice with lung cancer

Qi Li, Ke Hu<sup>✉</sup>, Si Tang, Li-Fang Xu, Yu-Chuan Luo

Department of Respiratory Medicine, Renmin Hospital of Wuhan University, 430060, China

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### ABSTRACT

**Objective:** To explore the anti-tumor activity of tanshinone IIA in combined with cyclophosphamide against Lewis mice with lung cancer and the effect on cellular immune function.

**Methods:** Lewis tumor cells were inoculated subcutaneously into the right armpit of mice in each group ( $n = 20$ ) to establish Lewis lung cancer mice model. After model establishment, mice in the model group were given normal saline by lavage, qd. Mice in treatment I group were given intraperitoneal injection of Tan IIA, 15 mg/kg, qd. Mice in treatment II group were given intraperitoneal injection of CTX, 25 mg/kg, qd. Mice in treatment III group were given intraperitoneal injections of Tan IIA and CTX, in which the administration method of Tan IIA was the same as in treatment I group, continuously for 2 weeks, and the dosage of CTX was the same as in treatment II group, 24 h after model establishment, every other day. Mice were sacrificed 2 weeks after establishment. The tumor tissues were collected to calculate the anti-tumor rate. Immunohistochemistry was used to detect the expressions of Bcl-2, Bax, VEGF, Angiostatin, and Endostatin. FCM was used to detect T lymphocyte subsets in spleen and liver of mice.

**Results:** The tumor weight in treatment I, II, and III groups was significantly lower than that in the model group ( $P < 0.05$ ). The tumor weight in treatment III group was significantly lower than that in treatment I and II groups ( $P < 0.05$ ). The anti-tumor rate in treatment II and III groups was significantly higher than that in treatment I group ( $P < 0.05$ ). Bcl-2 expression in the tumor tissues of treatment I, II, and III groups was significantly lower than that in the model group ( $P < 0.05$ ), while Bax expression was significantly higher than that in the model group ( $P < 0.05$ ). Bcl-2 expression in the tumor tissues of treatment I and II groups was significantly higher than that in treatment III group ( $P < 0.05$ ), while Bax expression was significantly lower than that in treatment III group ( $P < 0.05$ ).  $CD4^+$  and  $CD4^+/CD8^+$  in treatment I, II, and III groups were significantly higher than those in the model group ( $P < 0.05$ ).  $CD4^+$  in treatment III group was significantly higher than that in treatment I and II groups ( $P < 0.05$ ), while  $CD4^+/CD8^+$  was significantly higher than that in treatment II group ( $P < 0.05$ ). The comparison of  $CD8^+$  among each group was not statistically significant ( $P > 0.05$ ). NK cell activity in treatment I, II, and III groups was significantly higher than that in the model group ( $P < 0.05$ ). NK cell activity in treatment III group was significantly higher than that in treatment I and II groups ( $P < 0.05$ ).

**Conclusions:** Tan IIA in combined with CTX can down regulate Bcl-2 expression in lung cancer tissues, up regulate Bax expression, inhibit the neovascularization of tumor tissues, and enhance the immunological function, with a significant anti-tumor activity.

First author: Qi Li, Department of Respiratory Medicine, Renmin Hospital of Wuhan University, 430060, China.

Tel: +86 13429868677

E-mail: [liqi13429868677@163.com](mailto:liqi13429868677@163.com)

<sup>✉</sup>Corresponding author: Ke Hu, Department of Respiratory Medicine, Renmin Hospital of Wuhan University, 430060, China.

Tel: +86 18971035988

E-mail: [hukejx@163.com](mailto:hukejx@163.com)

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## 1. Introduction

Lung cancer is a common malignant tumor of respiratory system in the clinic, with an increasing morbidity due to the alterations of modern environment and life style, and has been one of the diseases which can severely threaten the human life and health [1–3]. The pathogenesis of lung cancer is not yet clear. Some scholars argue that its pathogenesis is closely associated with the long-term heavy smoking [4]. According to the statistics [5], in recent 50 years, the morbidity and fatality rate of lung cancer in males rank the first place among all malignant tumors. With the continuous maturity of radiotherapy and chemotherapy technology, the efficacy by medicine of lung cancer is improved greatly, but the total 5-year survival rate is only 10%–15%, with a poor long-term efficacy [6]. A large amount of researches verify that [7] operation, radiotherapy, and chemotherapy can produce a certain effect on the immunological function; therefore, how to effectively treat lung cancer and reduce the effect on immunological function as much as possible has been the hot tissue of clinical research. CTX, a common anti-tumor drug in the clinic, can intervene DNA and RNA functions in tumor cells, with a wide application [8,9]. Tan IIA can significantly resist the atherosclerosis, inhibit the platelet aggregation, kill various tumor cells, and promote their apoptosis [10]. In order to observe the anti-tumor activity of Tan IIA in combined with CTX against lung cancer mice and the effect on cellular immune function, C57BL/6J mice were selected to establish Lewis lung cancer mice model. After model establishment, mice were given Tan IIA and CTX to observe its anti-tumor activity against Lewis mice with lung cancer and the effect on T lymphocyte subsets.

## 2. Materials and methods

### 2.1. Experimental animals

A total of 80 male C57BL/6J mice (18–20 g), SPF, were obtained from Beijing HFK Bioscience Co. Ltd. The animals were housed under standard conditions of temperature ( $20 \pm 2^\circ\text{C}$ ) and relative humidity (36%), and had free access to diet and water. All the experiments were performed in the Animal Experimental Center of Wuhan University. The animals were treated and cared for strictly in accordance with the guidelines recommended by the Laboratory Animal Administration Rules. The experimental protocol was approved by our departmental ethics committee.

### 2.2. Drugs and chemicals

Tan IIA sulfonate sodium injection was purchased from Shanghai No. 1 Biochemical and Pharmaceutical Co., Ltd. (Specification: 10 mg/2 mL, Approval No. H31022558). CTX injection was purchased from Jiangsu Hengrui Medicine Co., Ltd. (Specification: 0.2 g/piece, Approval No. H32020857). Lewis lung cancer cell lines were provided by the Immunology Department of China Medical University. The anti-mice CD4 kits and anti-CD8 monoclonal antibodies (marked by PE) were purchased from BD Company, US. BBSDSC super clean bench was purchased from Shanghai Biboase. Olympus biological microscope (Japan), AnKe80-ZC desk centrifuge, 301-268.001 histotome, and Leica-DM2500 B microscope (German) were

also purchased. Image-pro plus 6.0 image processing software was adopted.

### 2.3. Model establishment

Lewis lung cancer cell lines were melted at  $37^\circ\text{C}$ , and centrifuged at 14 000 r/min. The supernatant was abandoned, and the suspension was diluted with cell concentration of  $1 \times 10^7/\text{mL}$  for preservation. The prepared cell suspension (0.2 mL) was inoculated subcutaneously into the right armpit of mice, with two generations. After inoculation, the mice were randomized into the model group, treatment I, II, and III groups with 20 mice in each group. After model establishment, mice in the model group were given normal saline by lavage, qd. Mice in treatment I group were given intraperitoneal injection of Tan IIA, 15 mg/kg, qd. Mice in treatment II group were given intraperitoneal injection of CTX, 25 mg/kg, qd. Mice in treatment III group were given intraperitoneal injections of Tan IIA and CTX, in which the administration method of Tan IIA was the same as in treatment I group, continuously for 2 weeks, and the dosage of CTX was the same as in treatment II group, 24 h after model establishment, every other day.

### 2.4. Observation indicators

Mice were sacrificed 2 weeks after establishment. The tumor tissues were collected to calculate the anti-tumor rate. Anti-tumor rate (%) = (average tumor weight in the model group – average tumor weight in the treatment group)/average tumor weight in the model group  $\times 100\%$ . Immunohistochemistry was used to detect the expressions of Bcl-2, Bax, VEGF, Angiostatin, and Endostatin. The spleen of mice in each group was collected under a sterile condition. The spleen cell suspension was prepared with cell concentration of  $1 \times 10^7/\text{mL}$ . FCM was used to detect T lymphocyte subsets in spleen of mice.

### 2.5. Statistical analysis

SPSS 16.0 software was used for the statistical analysis. The measurement data were expressed as mean  $\pm$  SD, and *t* test was used.  $P < 0.05$  was regarded as statistically significant.

## 3. Results

### 3.1. Comparison of the anti-tumor rate among four groups

The tumor weight in treatment I, II, and III groups was significantly lower than that in the model group ( $P < 0.05$ ). The tumor weight in treatment III group was significantly lower than that in treatment I and II groups ( $P < 0.05$ ). The anti-tumor rate in treatment II and III groups was significantly higher than that in treatment I group ( $P < 0.05$ ). The comparison of anti-tumor rate between treatment II and III groups was not statistically significant ( $P > 0.05$ ) (Table 1).

### 3.2. Comparison of Bcl-2 and Bax expressions in the tumor tissues among four groups

Bcl-2 expression in the tumor tissues of treatment I, II, and III groups was significantly lower than that in the model group

**Table 1**

Comparison of the tumor weight and anti-tumor rate among four groups (n = 20).

Groups	Tumor weight (g)	Anti-tumor rate (%)	Bcl-2	Bax
Treatment I group	2.49 ± 0.19 <sup>*#</sup>	25.8 <sup>*#</sup>	45.78 ± 5.42 <sup>*#</sup>	39.55 ± 3.92 <sup>*#</sup>
Treatment II group	1.21 ± 0.39 <sup>*#</sup>	50.1 <sup>*</sup>	35.82 ± 4.24 <sup>*#</sup>	43.47 ± 4.21 <sup>*#</sup>
Treatment III group	1.08 ± 0.48 <sup>*</sup>	58.3 <sup>*</sup>	25.15 ± 4.11 <sup>*</sup>	52.13 ± 5.36 <sup>*</sup>
Model group	3.34 ± 0.20	0.0	59.72 ± 3.48 <sup>#</sup>	31.85 ± 8.61 <sup>#</sup>

<sup>\*</sup>P < 0.05, when compared with the model group; <sup>#</sup>P < 0.05, when compared with treatment III group.

(P < 0.05), while Bax expression was significantly higher than that in the model group (P < 0.05). Bcl-2 expression in the tumor tissues of treatment I and II groups was significantly higher than that in treatment III group (P < 0.05), while Bax expression was significantly lower than that in treatment III group (P < 0.05) (Table 1).

### 3.3. Comparison of VEGF, Angiostatin, and Endostatin expressions in the tumor tissues among four groups

VEGF expression in treatment I, II, and III groups was significantly lower than that in the model group (P < 0.05), while Angiostatin and Endostatin expressions were significantly higher than those in the model group (P < 0.05). VEGF expression in treatment I and II groups was significantly higher than that in treatment III group (P < 0.05), while Angiostatin and Endostatin expressions were significantly lower than those in treatment III group (P < 0.05) (Table 2).

### 3.4. Comparison of the immunological function among four groups

CD4<sup>+</sup> and CD4<sup>+</sup>/CD8<sup>+</sup> in treatment I, II, and III groups were significantly higher than those in the model group (P < 0.05). CD4<sup>+</sup> in treatment III group was significantly higher than that in treatment I and II groups (P < 0.05), while CD4<sup>+</sup>/CD8<sup>+</sup> was significantly higher than that in treatment II group (P < 0.05). The comparison of CD8<sup>+</sup> among each group was not statistically significant (P > 0.05). NK cell activity in treatment I, II, and III groups was significantly higher than that in the model group (P < 0.05). NK cell activity in treatment III group was significantly higher than that in treatment I and II groups (P < 0.05) (Table 3).

## 4. Discussion

Lung cancer is a common malignant tumor of respiratory system in the clinic, occurring more in males than in females, and ranks the first place of malignant tumor death among the urban population [11]. Non-small cell lung cancer is a common type in the clinic, including large cell carcinoma, adenocarcinoma, and squamous-cell carcinoma, whose cell division is slower than other cancer cells, with later spreading and metastasis [12–15]. Due to its concealment, most non-small cell lung cancer is discovered on physical examination, but already progresses into the middle and advanced stage, with a low 5-year survival rate [16–18]; therefore, how to conduct an effective treatment is of great significance in improving the patients' survival.

Multiple factors are involved in the lung cancer pathogenesis which is not yet completely clarified [19]. Some scholars argue that [20] the imbalance of cell apoptosis and proliferation may be a main factor for developing lung cancer. Cell apoptosis is an initiative and physiological death mechanism of cell itself, and is regulated by the apoptosis inhibiting factor and pro-apoptosis factor. Bcl-2 and Bax belong to Bcl-2 family genes, and are closely associated the occurrence of various tumor tumors [21]. Bcl-2 a proto-oncogene cloned from the follicular B-cell non-Hodgkin lymphoma, and can inhibit the cell apoptosis, while Bax is a pro-apoptosis gene, and can resist Bcl-2 [22]. The results in the study showed that Bcl-2 expression in treatment I, II, and III groups was significantly lower than that in the model group (P < 0.05), while Bax expression was significantly higher than that in the model group (P < 0.05), suggesting that the 3 treatment protocols can down regulate Bcl-2 expression, and up regulate Bax expression. The results in the study also showed that Bcl-2 expression in the tumor tissues of treatment I and II

**Table 2**

Comparison of VEGF, Angiostatin, and Endostatin expressions in the tumor tissues among four groups (n = 20).

Groups	VEGF	Angiostatin	Endostatin
Treatment I group	22 525.08 ± 17 665.99 <sup>*#</sup>	30 758.38 ± 6 561.57 <sup>*#</sup>	26 022.40 ± 8 105.76 <sup>*#</sup>
Treatment II group	19 260.64 ± 5 414.20 <sup>*#</sup>	31 754.67 ± 7 142.30 <sup>*#</sup>	25 895.04 ± 13 523.83 <sup>*#</sup>
Treatment III group	11 512.88 ± 5 568.63	47 142.17 ± 10 476.41	28 009.05 ± 10 055.47
Model group	59 089.63 ± 17 740.77 <sup>#</sup>	13 319.41 ± 4 504.00 <sup>#</sup>	1 956.03 ± 2 348.28 <sup>#</sup>

<sup>\*</sup>P < 0.05, when compared with the model group; <sup>#</sup>P < 0.05, when compared with treatment III group.**Table 3**

Comparison of T lymphocyte subsets levels among four groups (%) (n = 20).

Groups	CD4 <sup>+</sup>	CD8 <sup>+</sup>	CD4 <sup>+</sup> /CD8 <sup>+</sup>	NK
Treatment I group	15.16 ± 1.79 <sup>*#</sup>	15.83 ± 5.41	1.17 ± 0.18 <sup>*</sup>	27.09 ± 0.06 <sup>*#</sup>
Treatment II group	12.95 ± 2.08 <sup>*#</sup>	16.65 ± 1.81	0.86 ± 0.24 <sup>*#</sup>	26.50 ± 0.05 <sup>*#</sup>
Treatment III group	22.25 ± 6.82 <sup>*</sup>	15.49 ± 4.65	1.37 ± 0.35 <sup>*</sup>	52.53 ± 0.16 <sup>*</sup>
Model group	09.11 ± 2.19	14.60 ± 3.30	0.62 ± 0.46 <sup>*</sup>	19.62 ± 0.04

<sup>\*</sup>P < 0.05, when compared with the model group; <sup>#</sup>P < 0.05, when compared with treatment III group.

groups was significantly higher than that in treatment III group ( $P < 0.05$ ), while Bax expression was significantly lower than that in treatment III group ( $P < 0.05$ ), indicating that Tan IIA in combined with CTX can promote the lung cancer cell apoptosis, with a more significant effect.

Some scholars argue that [22] when the tumor diameter is greater than 1 mm, the surrounding tissues are unable to provide the required nutrition for its growth. The sequential growth of tumor must depend on new blood vessels to maintain the nutrition supply, otherwise, degeneration will occur in the tumor cells. The tumor angiogenesis is a multi-step complicated process, including vascular endothelial cell proliferation and migration, and extracellular matrix degradation. VEGF is an important factor to promote the angiogenesis, while Angiostatin and Endostatin are the main inhibiting factors for angiogenesis [23]. The results in the study showed that VEGF expression in treatment I, II, and III groups was significantly lower than that in the model group ( $P < 0.05$ ), while Angiostatin and Endostatin expressions were significantly higher than those in the model group ( $P < 0.05$ ), suggesting that the 3 treatment protocols can inhibit the angiogenesis of tumor tissues. The results in the study also showed that VEGF expression in treatment I and II groups was significantly higher than that in treatment III group ( $P < 0.05$ ), while Angiostatin and Endostatin expressions were significantly lower than those in treatment III group ( $P < 0.05$ ), indicating that Tan IIA in combined with CTX can better effectively inhibit the angiogenesis of tumor tissues in order to reach the goal of anti-tumor. In the study, the anti-tumor rate in treatment III group was the highest, which is associated with the effective inhibition on the tumor angiogenesis by Tan IIA and CTX.

Some experiments verify that [24,25] the multiple immune escape mechanism formed in the growth process of cancer cells is an important factor for the continuous occurrence and development of tumor cells; therefore, the immunological function plays a vital role in the tumor treatment process. The results in the study showed that CD4<sup>+</sup> in treatment III group was significantly higher than that in treatment I and II groups ( $P < 0.05$ ), while CD4<sup>+</sup>/CD8<sup>+</sup> was significantly higher than that in treatment II group ( $P < 0.05$ ); moreover, NK cell activity in treatment III group was significantly higher than that in treatment I and II groups ( $P < 0.05$ ), indicating that this treatment protocol can effectively enhance the immunological function to strengthen the anti-tumor effect.

In conclusion, Tan IIA in combined with CTX can down regulate Bcl-2 expression in lung cancer tissues, up regulate Bax expression, inhibit the neovascularization of tumor tissues, and enhance the immunological function, with a significant anti-tumor activity.

## Conflict of interest statement

We declare that we have no conflict of interest.

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